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(54) **HSV VIRAL VECTOR**

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(2), (4) Date: **Jan. 28, 1997**

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514/44; 530/350; 536/23.1; 536/23.5

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536/23.5; 424/231.1, 199.1; 514/44; 530/350;
435/69.3, 320.1, 235.1

(57) **ABSTRACT**

A recombinant herpes simplex virus (HSV) viral vector genome which has substantially lost its transducing properties as a result of a DNA sequence change in the gene coding for Vmw65 protein and also contains an expressible heterologous gene inserted into a region of the HSV genome which is non-essential for the culture of the virus, the gene being under the control of the immediate early (IE1) gene enhancer of cytomegalovirus (CMV) and to the use of the recombinant HSV genome in therapy and vaccination.

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10 Claims, 1 Drawing Sheet

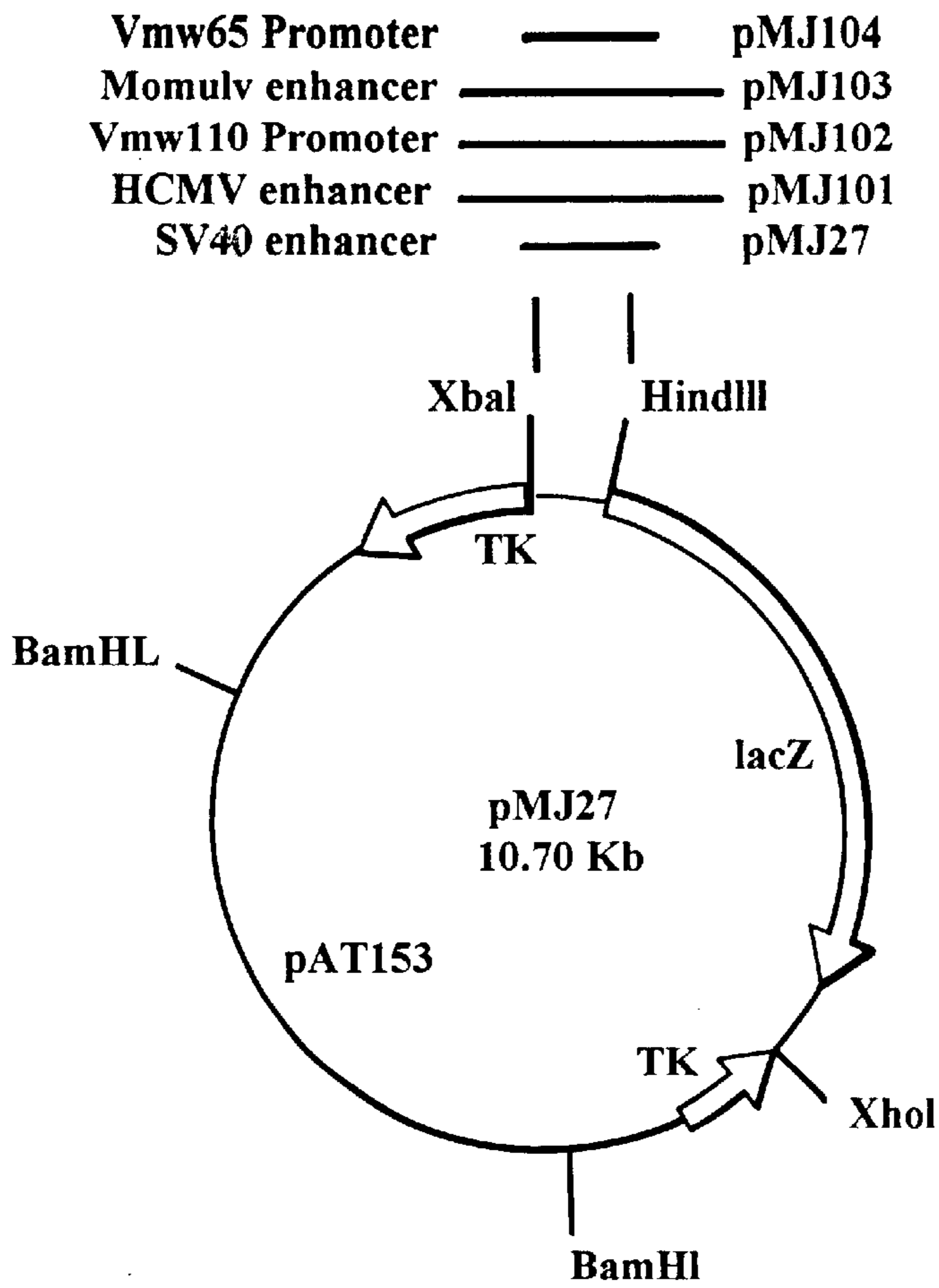


FIG. 1

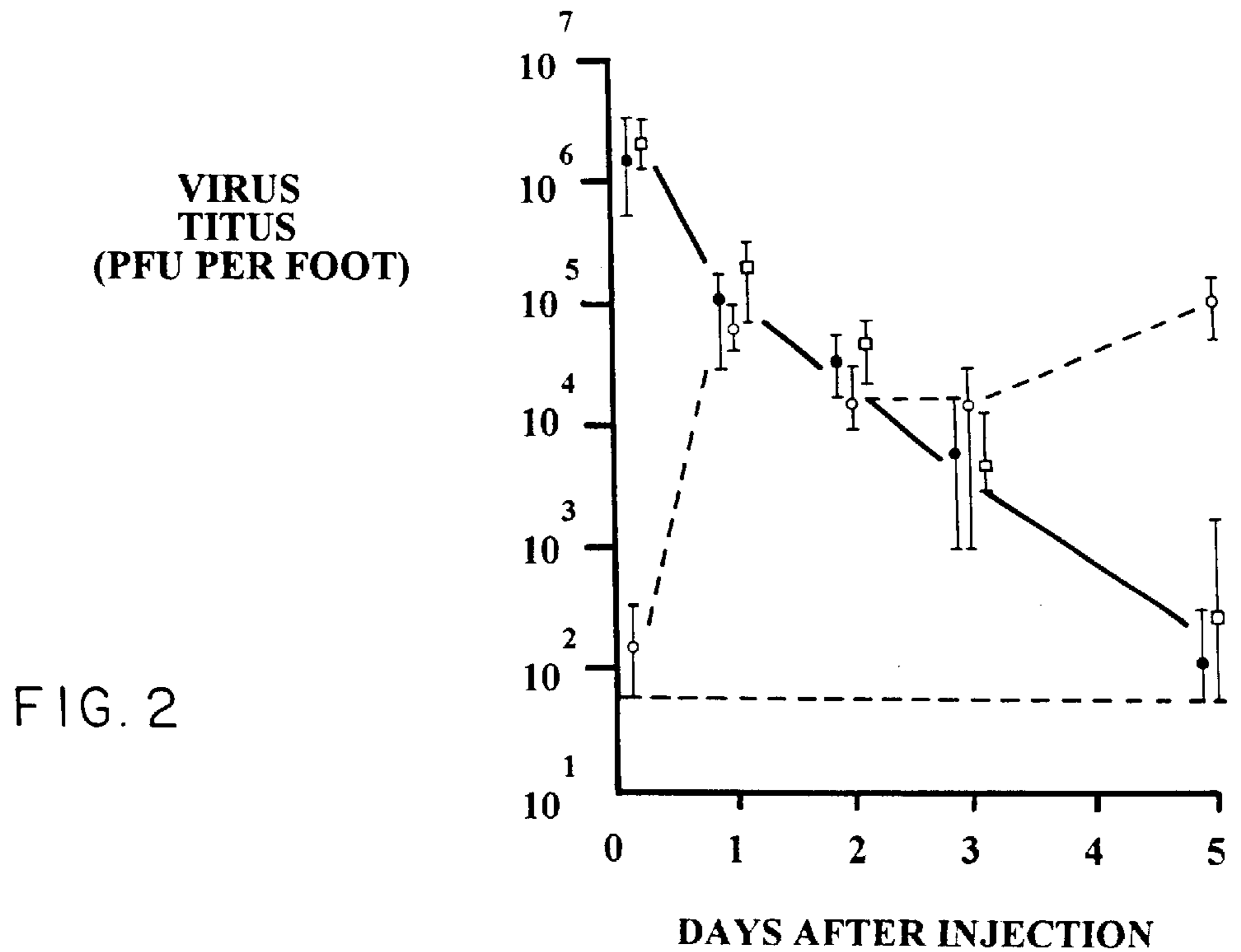


FIG. 2

HSV VIRAL VECTOR

The present application is a of 371 of PCT/GB95/01787, international filing date Jul. 27, 1995.

FIELD OF THE INVENTION

The present invention relates to a recombinant herpes simplex virus (HSV), especially type 1 (HSV-1) or type 2 (HSV-2) having a good ability to continuously express an inserted heterologous gene whilst the virus is at the same time maintained in its latent non-replicative state.

BACKGROUND OF THE INVENTION

A distinguishing feature of herpes virus infections is the ability to persist in the host for long periods in a non-replicative or latent state. Herpes simplex virus type 1 (HSV-1) establishes latent infection in human peripheral sensory ganglia and can reactivate to produce recurrent mucocutaneous lesions. Operationally, the pathogenesis of herpes virus infections can be divided into several distinct stages which can be studied individually in experimental animal models: acute viral replication, establishment of latency, maintenance, and reactivation. Following inoculation, HSV-1 replicates at the site of inoculation and is transported to sensory ganglia. Replication at the periphery or in sensory ganglia may increase the amount of virus that can establish latent infection. During latent infection, HSV-1 DNA can be detected in infected tissues but infectious virus cannot be detected. This latent state is often maintained for the life of the host. A variety of stimulac (such as fibrile illness and exposure to ultraviolet irradiation) can interrupt the latent state and cause the reappearance of infectious virus or reactivation.

Transcription of the HSV-1 immediate early (IE) genes is not detectable during latency. However, in tissue culture, IE gene expression is a pre-requisite for viral replication. Transcription of the IE genes is transinduced by a virion protein Vmw65 (transinducing factor) that is a component of the HSV-1 virion. Vmw65 does not bind directly to HSV-1 DNA but mediates transinduction by association with cellular proteins to form a complex which interacts with the IE regulatory element.

Ace et al (1989) report an HSV-1 mutant which contains a 12 bp insertion in the coding region of Vmw65 which is unable to transinduce IE gene expression, though the altered Vmw65 is incorporated into mature virions.

The inventor's previous patent specification WO91/02788 discloses a herpes simplex virus type 1 mutant which includes the mutation within the Vmw65 gene which removes the transinducing properties of the Vmw65 transactivator protein such that the virus remains in its latent state. In addition, a β -galactosidase marker gene under the control of the latency associated transcript (LAT) promoter is inserted into the thymidine kinase (TK) gene and expression of the heterologous gene during latency is observed.

It is an object of the present invention to provide an HSV viral vector having enhanced expression of the inserted heterologous gene during latency.

SUMMARY OF THE INVENTION

Generally speaking, the present invention is based on the discovery that enhanced long term expression during latency may be obtained by use of the IE1 gene enhancer of cytomegalovirus controlling the inserted heterologous gene.

Most specifically, the present invention provides a recombinant herpes simplex virus (HSV) viral vector genome which comprises;

- (i) a DNA sequence change in the gene coding for Vmw65 protein such as to substantially remove transinducing properties thereof; and
- (ii) an expressable heterologous gene inserted into a region of the HSV genome which is non-essential for culture of the virus, the gene being under the control of the immediate early 1 (IE1) gene enhancer of cytomegalovirus (CMV).

DETAILED DESCRIPTION OF THE INVENTION

The Vmw65 sequence change removes the transinducing properties thereof such that expression of HSV IE genes and therefore HSV viral replication in vivo, is substantially removed. The HSV vector is therefore constrained to remain in its latent state. The use of the IE1 CMV enhancer to control the inserted heterologous gene has been found to give excellent long term expression of the heterologous gene during latency. Experiments in mice using the inserted heterologous lacZ gene have showed continuous expression from the latent vector of up to five months. In contrast, use of other promoters such as HSV-1 Vmw110 and Vmw65, and the Moloney murine leukaemia virus enhancer have been found not to give long term expression during latency.

The structure of the human cytomegalovirus (HCMV) enhancer is discussed in Stinski and Roehr (1985). The IE1 enhancer is the promoter-regulatory region upstream of the major immediate early gene of human cytomegalovirus. This enhancer region upstream of the IE1 gene consists of a series of different repeat sequences distributed up to -509 bp from the site for the initiation of transcription. Within this enhancer are a set of inducing sequences. Certain of the sequences within the enhancer region are non-essential and do not effect the level of expression obtained, whilst other sequences promote downstream expression.

The CMV enhancer is generally that derived from human cytomegalovirus (HCMV) and the immediate early 1 (IE1) nomenclature applies particularly to that virus. However, the analogous enhancer from other types of CMV, such as mouse, rat, equine, simian, and guinea pig CMV, may also be employed.

The present invention primarily envisages the use of the entire CMV IE1 enhancer sequence. Indeed in a preferred embodiment of the invention a larger sequence extending to -730 bp and including the entire CMV IE1 enhancer was employed. However, it is clearly within the ambit of the skilled man to modify the naturally occurring enhancer sequence without departing from the general scope of the present invention. Thus, the present invention is concerned not only with the use of the entire CMV IE1 enhancer sequence but also with variations in that sequence, either by insertion, deletion or substitution such that the enhancer properties are not substantially affected.

Other promoter sequences, such as the LAT (latency associated transcript) promoter, may be included upstream of the inserted heterologous gene and HCMV enhancer, but these have not been found to offer any particular advantage according to the present invention.

The position and size of the DNA sequence change in the gene coding for Vmw65 protein is significant, since it is necessary to substantially remove the transinducing properties of the Vmw65 protein (and thereby prevent in vivo replication of the virus and consequent illness of the patient), whilst at the same time retaining the structural properties of the protein required to successfully assemble the complete virion when the virus is cultured. The viral vector of the

present invention must be capable of replication under culture conditions so as to be able to produce sufficient quantities of the mutant virus for use, but at the same time the virus should be incapable of replication in vivo. Preferably, the DNA sequence change is achieved by a transition (purine to purine or pyrimidine to pyrimidine) or transversion (purine to pyrimidine or vice versa) alteration of 1–72 base pairs, an oligonucleotide insert of 3–72 base pairs or a deletion of 3–72 base pairs, at a position between amino acids 289 and 480 (especially 289 and 412) of the Vmw65 protein.

The recombinant HSV may be of type HSV-1 or HSV-2 or may be an intertype recombinant between HSV-1 and HSV-2 which comprises nucleotide sequences derived from both types. The recombinant HSV genome will generally be contained in a mutant HSV virus.

HSV has the ability to infect many tissue types and therefore in principle the viral vector of the present invention may be used as a vector directed against a wide variety of cell types. Latency in HSV infection tends to be established within neuronal cells, though it is possible that expressed gene products may translocate from their original point of production. The viral vector of the present invention is thus particularly useful for delivering expressible heterologous genes into neuronal cells. The genes may deliver a therapeutic effect or may deliver an antigenic protein for stimulating the production of antibodies when used as a vaccine. The therapeutic gene is generally a gene associated with a neurological genetic deficiency i.e. it compensates for an inherited or acquired genetic deficiency. Examples of such therapeutic genes include:

- (a) human, rat or mouse tyrosine hydroxylase genes 1, 2 or 3, which are relevant to the alleviation of symptoms of Parkinson's disease;
- (b) human, rat or mouse nerve growth factor (e.g. the beta subunit) for treatment of Alzheimer's disease and Parkinson's disease;
- (c) human, rat or mouse hypoxanthine-guanine phosphoribosyl transferase gene for the treatment of Lesch-Nyhan disease;
- (d) human beta-hexosaminidase alpha chain gene, for the treatment of Tay-Sachs and Sandhoff's diseases; and
- (e) human immunodeficiency virus (HIV) nef gene, for the control of neurological symptoms in HIV-positive individuals.

In particular, the in situ expression of tyrosine hydroxylase by the HSV viral vector of the present invention may help alleviate the symptoms of Parkinson's disease. Tyrosine hydroxylase is a crucial enzyme in the synthesis of dopamine. Deficiency of dopamine is the major cause of symptoms in Parkinson's disease, and current treatment involving the administration of L-dopa gives only—short-lived respite.

The heterologous gene may be inserted into any region of the viral genome which is non-essential for the culture of the virus, i.e. replication of the virus outside the body, particularly in tissue culture. In particular, the insertion of the heterologous gene could be made in the coding sequences or in the flanking control regions of one or more of the following HSV-1 genes:

1. The thymidine kinase gene (the UL23 gene); which is the preferred choice since thymidine kinase is important for pathogenicity of HSV, so that deactivation of its gene may reduce potential pathogenicity of the mutant vector.
2. The RL1 gene
3. The RL2 gene (otherwise named the IE110 gene)

4. The locus encoding the latency associated transcripts
 5. The UL2 gene (otherwise named the Uracil-DNA glycosylase gene)
 6. The UL3 gene
 7. The UL4 gene
 8. The UL10 gene
 9. The UL11 gene
 10. The UL13 gene
 11. The UL16 gene
 12. The UL20 gene
 13. The UL24 gene
 14. The UL40 gene (otherwise named the gene encoding the small subunit of ribonucleotide reductase)
 15. The UL41 gene (otherwise named the virion host shutoff factor gene)
 16. The UL43 gene
 17. The UL44 gene
 18. The UL45 gene
 19. The UL46 gene
 20. The UL47 gene
 21. The UL50 gene (otherwise named the dUTPase gene)
 22. The UL55 gene
 23. The UL56 gene
 24. The US1 gene (otherwise named the IE68 gene)
 25. The US2 gene
 26. The US3 gene (otherwise named the protein kinase gene)
 27. The US4 gene (otherwise named the glycoprotein G gene)
 28. The US5 gene
 29. The US7 gene (otherwise named the glycoprotein I gene)
 30. The US8 gene (otherwise named the glycoprotein E gene)
 31. The US9 gene
 32. The US10 gene
 33. The US11 gene
 34. The US12 gene (otherwise named the IE12 gene)
- The UL, US and RL nomenclature system given above is a systematic one, but certain common names of genes are also included.

Another aspect of the present invention relates to the use of the recombinant HSV viral vector genome comprising an appropriate expressible therapeutic gene in the therapy of disease, particularly diseases due to or associated with genetic deficiency. The viral vector may also be used as a vaccine to deliver an antigenic protein.

A further aspect of the present invention relates to a pharmaceutical composition for administering the viral vector comprising the viral vector in admixture with a pharmaceutically acceptable carrier. Generally, the composition will be formulated for parenteral administration—usually by injection—in an appropriate acceptable carrier such as pyrogenic isotonic saline.

The present invention is hereafter further described by way of example only, the insertion of a gene (the lacZ gene coding for β -galactosidase) into the viral vector of the present invention. The lacZ gene is inserted in order to demonstrate the technology, since the presence of the gene is easily detectable. However, for therapeutic or other applications, a heterologous gene would be inserted in an

analogous manner; or the lacZ gene could be directly replaced by another heterologous gene.

FIGURES

FIG. 1 is a diagrammatic representation of the plasmids used to construct the viruses used in this study. In1853 is a mutant virus according to the present invention, whilst the others are for comparison purposes. Comparison mutants in1863 and in1891 also contain the HCMV enhancer but these are positive for Vmw65 and TK respectively.

FIG. 2, titres of viruses after injection into mouse footpads. Virus was assayed in extracts of feet after injection of 8×10^7 pfu of in1853 (\square), in1863 (\bullet) or 1×10^5 pfu of 1814R (\circ). The means and range of titres from four animals are shown. The level of detection was 50 pfu per foot.

EXAMPLES SECTION

Materials and Methods

Cells

BHK clone 13 cells (Macpherson and Stoker, 1962) were grown in Eagle's Medium (Glasgow modification) supplemented with 10% newborn calf serum, 10% tryptose phosphate broth, 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin (ETC₁₀).

Viruses

The HSV-1 strain 17 mutant in1814 containing a 12 bp insertion in the coding sequences for Vmw65, and the rescued 'revertant' 1814R, have been described previously (Ace et al, 1989).

To prepare HSV recombinants (summarised in Table 1) containing the *E. coli* lacZ gene inserted within the TK gene, plasmid pMJ27 was first constructed. Plasmid pGX166 (kindly supplied by V. G. Preston) is the cloned HSV-1 strain 17 BamHI p fragment modified by the insertion of an XhoI linker at the SacI site within the TK coding sequences. The HindIII site within the pAT153 vector sequences of pGX166 was destroyed by cleavage, end-filling with Klenow enzyme and religation, to yield pGX166 ΔH3 . The *E. coli* lacZ gene plus simian virus 40 (SV40) promoter and enhancer was excised from plasmid FJ3 (Rixon and McLauchlan, 1990) as a 4073 bp BamHI/XbaI fragment and cloned between the BamHI and XbaI sites of pUC18 (previously modified by insertion of an XhoI linker into the SmaI site) to yield pUC18lacZ. The lacZ gene plus SV40 promoter and polyadenylation signals were excised from pUC18lacZ as a Sall/XhoI fragment and cloned into the XhoI site of pGX166 ΔH3 . A plasmid in which the direction of lacZ transcription was opposite to that of TK was selected and designated pMJ27. Plasmid pMJ27 contains unique XbaI and HindIII sites flanking the SV40 promoter plus enhancer, and has a unique XhoI site downstream of the SV40 polyadenylation signal.

The human cytomegalovirus (HCMV) Towne strain enhancer was cloned as a 760 bp Sau3AI fragment (Stinski and Roehr, 1985) from plasmid pHD101-4 (kindly provided by E. Blair) into the BamHI site of pUC18 (from which the SphI site had been inactivated by Klenow treatment and religation), excised as an EcoRI (end-filled)/HindIII fragment and cloned between the XbaI(end-filled) and HindIII sites of pMJ27 in the appropriate orientation, to yield plasmid pMJ101. The HSV-1 Vmw110 promoter was excised as an 836 bp BbvI(end-filled)/SacI fragment from pJR3 (Everett, 1984) and cloned between the SphI (Klenow-

treated) and SacI sites of pUC18. The promoter was then removed as an EcoRI(end-filled)/HindIII fragment and cloned between the XbaI(end-filled) and HindIII sites of pMJ27, to yield plasmid pMJ102. The Moloney murine Leukaemia virus (Momulv) promoter plus enhancer was cloned as a 760 bp EcoRI/SmaI fragment, modified by the addition of BamHI linkers at each end (Lang et al, 1983) into the BamHI site of pUC18 (without the SphI site, as described above). The Momulv enhancer was then excised as an EcoRI(end-filled)/HindIII fragment and cloned between the XbaI(end-filled) and HindIII sites of pMJ27 to yield pMJ103. The Vmw65 promoter was excised as a 380 bp TaqI/EcoRV fragment from pMC1 (Campbell et al, 1984) and cloned between the SphI (Klenow treated) and AccI sites of pUC18. The promoter was excised as a XbaI/HindIII fragment and cloned between the XbaI and HindIII sites of pMJ27, to yield pMJ104. The structures of the pMJ plasmids are shown in FIG. 1.

For insertion into the UL43 gene, plasmid p35 (kindly supplied by Dr. C. A. MacLean; MacLean et al, 1991) was modified by insertion of an oligonucleotide linker, providing XbaI and XhoI sites, into the NsiI site within the UL43 coding sequences. Plasmid pMJ101 was partially digested with XbaI, digested with XhoI and the larger fragment spanning the β -gal gene plus HCMV enhancer was cloned between the XbaI and XhoI sites introduced into p35.

Plasmids were cleaved with ScaI and co-transfected into BHK cells with in1814 DNA. Progeny viruses containing lacZ inserts were identified by the development of blue plaques in the presence of 5-bromo-4-chloro-3-indolyl- β -D galactopyranoside (X-gal) and purified by three rounds of enrichment for lacZ-containing viruses. Final plaque isolates were grown as small scale cultures and DNA was purified from infected cells. For insertions in the TK gene, DNA was cleaved with EcoRI and analysed by Southern hybridization, using a ³²P radiolabelled 2.4 kbp EcoRI fragment spanning the TK gene as probe. Insertion of lacZ disrupts the 2.4 kbp fragment, yielding species of 1.0 kbp and 2.0 or 5.0 kbp, depending upon the structure of the lacZ-containing plasmid used. Virus isolates showing the correct pattern and lacking detectable hybridization to a 2.4 kbp fragment were grown as stocks.

For insertion into UL43, DNA was cleaved with EcoRI plus BamHI and probed with radiolabelled p35. Insertion of lacZ disrupts the 5.14 kbp EcoRI/BamHI fragment, yielding smaller species. An isolate with the appropriate restriction pattern was grown as a virus stock.

Rescue of the insertion at the Vmw65 locus was achieved by co-transfecting pMC1 with DNA from lacZ-containing viruses. Viral DNA was cleaved with BamHI, probed with pMC1 and isolates lacking a BamHI site within the Vmw65 coding sequences were grown as virus stocks.

Virus titres were determined on BHK cells in the presence of 3 mM hexamethylene bisacetamide (McFarlane et al, 1992).

Inoculation of Mice

Viruses were inoculated subcutaneously into the right rear footpad of 3 to 4 week old male BALB/c mice. Each mouse received 0.025 ml containing approximately 8×10^7 pfu of virus, diluted in ETC₁₀. At various times after inoculation, lumbar dorsal root ganglia (DRG) from spinal levels 2 to 6 (L2 to L6), and feet, were removed by dissection and either used for reactivation studies, stored at -70° for virus assay or fixed in 4% formaldehyde at 4° for 1 h for use in situ β -gal assay.

β -gal Assays

For quantitative assay of β -gal levels, ganglia were homogenised in phosphate buffered saline (PBS) and cells pelleted by centrifugation. Pellets were resuspended in 50 μ l of lysis buffer (10 mM Tris/HCl, pH7.5; 2 mM MgCl₂; 10 mM NaCl; 0.1% Nonidet P40), maintained at 4° C. in lysis buffer for 5 min, mixed by vortexing and stored at -70° C. After thawing, 20 μ l was assayed in a 100 μ l reaction mixture containing 25 mM Tris/HCl, pH7.5; 125 mM NaCl; 2 mM MgCl₂; 13 mM β -mercaptoethanol and 0.3 mM 4-methylumbelliferyl- β -D-galactoside. After incubation at 37° for 2 h, 3 μ l 20% trichloroacetic acid was added. After 5 min at 4° C., samples were centrifuged at 13,000 g for 5 min. Portions (100 μ l) of the supernatant were added to a reagent containing 133 mM glycine and 83 mM Na₂CO₃, pH 10.7 and fluorescence measured in a TKO 100 mini-fluorometer (Hoeffer). Protein concentrations of ganglion extracts were determined using a Sigma protein assay kit.

For in situ assay of β -gal, fixed DRG were washed twice with PBS, incubated at 37° C. in a mixture containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl₂, 0.02% Nonidet P40, 0.02% sodium deoxycholate and 1 mg/ml X-gal in PBS. Each reaction was performed on a glass slide in a volume of 150 μ l, with a 22x40 mm coverslip sealed over the DRG with wax. At 3, 24 or 48h the number or blue cells was counted and the DRG photographed using a Zeiss Axioskop microscope with MC80 camera attachment. Sealed preparations were turned over to check that β -gal containing cells which could not be seen by focusing through the preparation were not missed.

Reactivation

DRG were excised and incubated for 3 days at 37° C. in ETC₁₀ plus 10% foetal calf serum and 2.5 μ g/ml amphotericin B, conditions previously used to induce reactivation of latent TK⁺ viruses (Ecob-Prince et al, 1993b; Ecob-Prince and Hassan, 1994). Explanted DRG were then fixed and stained for the presence of β -gal.

Virus Assay

Feet or pooled L3/L4/L5 DRG were homogenized, frozen and thawed twice, and sonicated prior to assay for virus, as described previously (Robertson et al., 1992).

In Situ Hybridization (ISH)

After the in situ assay for β -gal, the fixed explanted DRG from each mouse were embedded together in one block of paraffin, serially-sectioned and the percentage of neurons which expressed LATs was determined by ISH (Ecob-Prince et al, 1993a).

Example 1

Footpad Inoculation of in1853 and in1863

Mutants in1853 and in1863 were injected into mice via the footpad. Their replication in the footpad and within the DRG was measured and β -gal expression was examined in DRG at various times after inoculation. The viruses possessed insertions of the lacZ gene controlled by the HCMV enhancer, at the TK locus, and thus were expected not to replicate in neurons. Injection of in1853 or in1863 at 8x10⁷ pfu resulted in no animal death and only a transient inflammation of the footpad in approximately 10% of mice. High virus titres were observed in the footpad at 2 hr post infection (pi) of either in1853 or in1863 (FIG. 2), but the

amount of virus detected decreased over a period of 5 days, suggesting there was a failure to replicate at the periphery and demonstrating that the absence of functional Vmw65 did not affect titres of these TK³¹ viruses in the foot. In contrast, the titres of 1814R following injection at 10⁵ pfu per mouse were maintained within the foot, as described previously (Robertson et al, 1992) When DRG were examined at 5 days post infection (pi), no virus (<5 pfu in total) was detected in pooled L3/L4/L5/L6 ganglia of in1853 and in1863-infected mice, whereas virus (90-4200 pfu) was detected in all animals infected with 1814R.

Example 2

Expression of β -gal in DRG Neurons

At various times after inoculation of mice or after explanation of latently-infected ganglia, individual DRG were scored by in situ assay for the number of β -gal containing cells. Invariably, only L3, L4 and L5 ganglia were positive, with the highest number usually in L4. Results are presented in Table 2 as a summation of the values from these ganglia, as suggested by Schmallbruch (1987). In preliminary experiments, DRG from 48 animals were scored after 3 or 21 h incubation in the β -gal reaction mixture, and the number found after 3 h was, on average, 56% of that detected after 21 h. No further increase was observed if the reaction time was extended to 48 h, so counts were thereafter performed following incubation of ganglia for 24 h.

The pattern of β -gal distribution in individual neurons varied, as observed by others (Ho and Mokarski, 1989; Dobson et al., 1990). We recognised a heavy homogenous stain which often extended to the axon of the neuron, a pale homogenous pattern confined to the cell body, a speckled distribution in which 5 to 20 foci of stain were observed in each cell, and a pale-speckled pattern in which the foci were superimposed on a uniformly coloured background. To investigate the relationship between these patterns, DRG were photographed after 3 h or 21 h in β -gal reaction mixture, and changes in the staining pattern of individual neurons observed. Some neurons initially classed as pale became heavy, and some speckled became pale-speckled, but there was little evidence that cells with a speckled pattern at 3 h became homogeneously stained by 21 h. The two basic patterns of staining, homogenous and speckled, thus appeared to represent distinct distributions of enzyme rather than be the result of a simple quantitative difference.

The numbers of neurons in DRG infected with in1853 or in1863 which contained β -gal were similar at either 3 or 5 days pi, with the homogenous staining pattern predominating (Table 2). Very few neurons of speckled appearance were detected at 3 days pi, but cells of this type were evident by 5 days. At 1 month pi, when latency was established, the number of β -gal containing neurons had fallen to about half of the value at 3 days pi, and the staining was predominantly speckled (Table 2). However, this predominantly speckled pattern and the number of β -gal containing cells was thereafter stable through 2 and 3 months pi and was also found in DRG at 5 months pi with in1853. These two characteristics of β -gal expression did not appear to be influenced by the presence (in1863) or absence (in1853) of functional Vmw65 in the inoculated virus. The homogenous (usually heavy) pattern was found to predominate during lytic replication (3 and 5 days pi) and reactivation following explanation, whereas the speckled pattern predominated at all times (1, 2, 3 and 5 months pi) during latency.

To investigate whether the long-term detection of β -gal in mice latently-infected with in1853 and in1863 was due to

specific features of the HCMV enhancer, additional virus mutants (Table 1) of the same basic design but with different promoters, regulating β -gal expression, in the presence or absence of a mutated Vmw65 gene, were tested. Mutants in1852 and in1862 contain the Momulv enhancer, whereas in1854 and in1864 contained the IE Vmw65 gene promoter. These viruses were injected into mice and β -gal expression examined in DRG at 3 days and 1 month pi, and after 3 months pi either directly or after explantation (Table 3). In addition, LAT-positive neurons were quantified by ISH at 3 months pi (Table 4). All mutants expressed β -gal at 3 days pi, with a preponderance of homogeneously stained neurons. The numbers of positive neurons were lower than found after infection with in1853 or in1863, particularly in the case of in1854 and in1864. No significant difference was observed between viruses possessing Vmw65 mutations (in1852 and in1854) and their rescued progeny (in1862 and in1864 respectively). However, no β -gal containing cells were detected during latency after infection with these viruses or with in1855, but explantation resulted in the appearance of 10–20, mainly homogeneously stained, neurons. When establishment of latency was assessed by ISH, equivalent number of LAT-expressing neurons were detected at 3 months post injection with all the viruses (Table 4).

To investigate whether persistence of β -gal after infection with in1853 or in1863 was due to the use of the TK gene as an insertion site, mutant in1891 (in which the UL43 gene was chosen as the insertion site) was constructed and tested. Mutants unable to express UL43 are able to establish, maintain and reactivate from latency following injection of mouse pinna (MacLean et al, 1991). At 3 days pi, in1891 caused an apparently more widespread infection of ganglia involving groups of cells stained, thus counting of β -gal containing neurons at 3 days pi was not attempted. Nonetheless, during latency and reactivation, the pattern of β -gal expression was very similar to that observed after injection of in1853 (Table 5). The presence of enzyme was thus a consequence of the use of the HCMV enhancer rather than the site of insertion or the TK⁻ phenotype of in1853 and in1863.

The HCMV enhancer is one of the strongest known promoters, and visualisation of stained ganglia suggested that in1853 and in1863 expressed β -gal to greater extents than the other TK⁻ viruses during acute infection. To quantify enzyme levels, two groups of 9 mice were injected with in1853 or in1855, the most active mutant in lytic infection based on visual observation of β -gal containing cells. At 3 days pi L3, L4 and L5 DRG from the left (uninjected) or right (injected) sides of each of 6 animals were removed by dissection and assayed individually for β -gal levels. The average levels of activity found in DRG of the left side of each group were remarkably similar (Table 6). Compared to these background levels, DRG from mice infected with in1853 were 6.6 times higher, and those from mice infected with in1855 were 1.8 times higher, than background. An in situ β -gal assay of DRG from 3 other mice for each virus yielded an average of 122 (for in1853) and 66 (for in1855) stained neurons per mouse, similar to the values obtained previously (Tables 2, 3 and 5), confirming that (even allowing for the difference in numbers of β -gal positive cells) in1853 expressed more β -gal than in1855.

The results present here demonstrate that the presence or absence of functional Vmw65 in the virus did not alter any aspect of heterologous lacZ gene expression or of latency. Viruses which were Vmw65 negative (in1852, in1853 and in1854) could not be distinguished from those which were Vmw65 positive (in1862, in1863 and in1864) in this respect.

Those mutant viruses which contained the HCMV enhancer (in1853, in1863 and in1891) showed good long-term expression of the inserted heterologous lacZ marker gene during latency. However in1863 and in1891 being Vmw65 positive and TK positive respectively are virulent and thus unsuitable for use as a viral vector in vivo.

TABLE 1

Characteristics of the viruses used.			
Virus	Promoter for lacZ	Location of construct	Phenotype *
in1853	HCMV	TK	TK ⁻ /Vmw65 ⁻
in1863	HCMV	TK	TK ⁻ /Vmw65 ⁺
in1852	Momulv	TK	TK ⁻ /Vmw65 ⁻
in1862	Momulv	TK	TK ⁻ /Vmw65 ⁺
in1854	Vmw110	TK	TK ⁻ /Vmw65 ⁻
in1864	Vmw110	TK	TK ⁻ /Vmw65 ⁺
in1855	Vmw65	TK	TK ⁻ /Vmw65 ⁻
in1891	HCMV	UL43	TK ⁺ /Vmw65 ⁻

* Vmw65⁻ and Vmw65⁺ refer to transactivating activity.

TABLE 2

The proportions of cells positive for B-gal which have different patterns of staining at different times after infection or explantation of the DRG.						
Time after infection or explantation	Total* (L3, 4, 5)	Heavy	Pale	Speckled	Pale/Speckled	
in1853: 3 dpi	103	82 (79%)	10 (9%)	8 (8%)	3 (3%)	
5 dpi	88	21 (24%)	36 (41%)	22 (25%)	9 (10%)	
1 mpi	37	3 (8%)	3 (7%)	27 (74%)	4 (11%)	
2 mpi	65	9 (14%)	16 (25%)	28 (43%)	12 (18%)	
3 mpi	49	3 (6%)	2 (4%)	38 (78%)	6 (12%)	
5 mpi	66	1 (1%)	0	55 (86%)	10 (13%)	
3 dpe	78	29 (37%)	32 (40%)	14 (18%)	4 (5%)	
in1863: 3 dpi	70	44 (63%)	10 (14%)	12 (17%)	4 (6%)	
5 dpi	65	12 (18%)	25 (39%)	22 (33%)	6 (10%)	
1 mpi	49	3 (7%)	1 (3%)	40 (82%)	4 (8%)	
2 mpi	51	2 (4%)	4 (6%)	43 (84%)	3 (6%)	
3 mpi	57	3 (6%)	6 (10%)	37 (65%)	11 (19%)	
3 dpe	78	28 (36%)	31 (40%)	16 (20%)	3 (4%)	

*all values are an average of at least 3 animals at each time

dpi, days post infection

mpi, months post infection (latent virus)

dpe, days post explantation (reactivating virus)

TABLE 3

The proportions of cells positive for b-gal which show different patterns of staining at different times after infection or explantation of the DRG infected with viruses containing lacZ controlled by different promoters in the TK gene location.

Time after infection or explantation	Total* (L3, 4, 5)	Heavy	Pale	Speckled	Pale/speckled
3 dpi: in1862	41	9 (22%)	23 (56%)	8 (20%)	1 (2%)
in1852	57	9 (15%)	33 (58%)	12 (21%)	3 (6%)
in1864	18	5 (25%)	13 (75%)	zero	zero
in1854	22	6 (27%)	16 (73%)	zero	zero
in1855	77	22 (28%)	54 (70%)	1 (2%)	zero
1 mpi	zero	zero	zero	zero	zero
All viruses					
3 mpi	zero	zero	zero	zero	zero
All viruses					
3 dpe: in1862	11	1 (9%)	10 (91%)	zero	zero
in1852	12	3 (25%)	7 (50%)	1 (8%)	1 (9%)
in1864	10	2 (20%)	7 (70%)	1 (10%)	zero

TABLE 3-continued

The proportions of cells positive for b-gal which show different patterns of staining at different times after infection or explantation of the DRG infected with viruses containing lacZ controlled by different promoters in the TK gene location.

Time after infection or explantation	Total* (L3, 4, 5)	Pale/speckled			
		Heavy	Pale	Speckled	Pale/speckled
in1854	16	4 (25%)	12 (75%)	zero	zero
in1855	17	2 (12%)	15 (88%)	zero	zero

*values are the average of 3 mice (3 dpi, 1 mpi) or 2 mice (3 mpi, 3 dpe)

TABLE 4

A comparison of the total number of neurons in L3, L4 and L5 DRG which contained either β -gal or LATs at 3 months pi.

Virus	β -gal positive neurons	LAT-positive neurons
in1862	0	192
in1852	0	161
in1864	0	117
in1854	0	155
in1853	49	143
in1863	57	218
in1891	32	172
in1855	0	224

TABLE 5

The proportions of cells positive for β -gal which showed different patterns of staining at different times after infection or following explantation of the DRG infected with viruses containing HCMV-lacZ in different locations.

Time after infection or explantation	Total (L3, 4, 5)	Pale/speckled				
		Heavy	Pale	Speckled	Pale/speckled	
in1853:	3 dpi	110*	35 (32%)	65 (59%)	9 (8%)	1 (1%)
	1 mpi	23*	zero	1 (3%)	17 (77%)	5 (20%)
	3 mpi	43†	zero	3 (8%)	38 (89%)	2 (3%)
in1891	3 dpe	75†	30 (40%)	32 (42%)	8 (10%)	6 (8%)
	1 mpi	15*	<1 (4%)	3 (19%)	10 (69%)	1 (8%)
	3 mpi	32†	zero	4 (13%)	21 (65%)	7 (22%)
	3 dpe	67†	29 (42%)	30 (44%)	8 (13%)	zero

*average value for 3 mice (9DRG) per time point
†average value for 2 mice (6DRG) per time point

TABLE 6

Assay for β -gal in DRG 3 days pi with different virus constructs.

Virus	Animal no.	Units of β -gal per μ g protein		
		Left*	Right*	Right/Left
in1853	1.	191	1562	7.7
	2.	325	1767	5.4
	3.	350	2828	8.1
	4.	196	1161	5.9
	5.	219	1238	5.7
	6.	126	858	6.8
in1855	average	235	1569#	6.6
	7.	212	322	1.5
	8.	426	869	2.0
	9.	445	653	1.5
	10.	157	223	1.4
	11.	107	307	2.9

TABLE 6-continued

Assay for β -gal in DRG 3 days pi with different virus constructs.

Virus	Animal no.	Units of β -gal per μ g protein		
		Left*	Right*	Right/Left
	12.	119	174	1.5
	average	244	425##	1.8

*pooled values of L3 + L4 + L5 DRG
#right significantly > left, p < 0.01
##right significantly > left, p < 0.05

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- What is claimed is:
1. A mutant recombinant herpes simplex virus (HSV) viral vector genome comprising:
 - (i) a mutation in a gene encoding a Vmw65 protein, the mutation being selected from the group consisting of: a transition or transversion alteration of 1–72 base pairs, an insertion of 3–72 base pairs, and a deletion of 3–72 base pairs, said mutation being at a position within said gene encoding an amino acid between amino acids 289 and 480 of the protein, wherein said mutant genome encodes a mutant Vmw65 protein lacking the ability to transinduce HSV-1 immediate early gene expression, whereby in vivo replication of the viral vector is prevented, wherein the ability of a virion produced from the viral vector genome to assemble is retained; and
 - (ii) an expressible heterologous gene inserted into a region of the HSV genome which is non-essential for

culture of the virus in vitro, the heterologous gene being under the control of an immediate early 1 (IE1) gene enhancer of cytomegalovirus (CMV), wherein said heterologous gene is expressed during latency of said virus;

wherein said mutation is a 12 base pair insertion at a in 1814 mutation site at a position within the Vmw65 gene encoding amino acid 379.

2. A recombinant HSV genome according to claim 1 wherein the CMV gene enhancer is the human CMV enhancer.

3. A recombinant HSV genome according to claim 1 wherein the CMV gene enhancer contains sequence variations such that the enhancer properties are not affected.

4. A recombinant HSV genome according to claim 1 wherein said HSV is HSV-1.

5. A recombinant HSV genome according to claim 1 wherein the expressible heterologous gene is selected from the group consisting of

- (a) human, rat or mouse tyrosine hydroxylase genes 1, 2 or 3;
- (b) human, rat or mouse nerve growth factor;
- (c) human, rat or mouse hypoxanthine-guanine phosphoribosyl transferase gene;

(d) human beta-hexosaminidase alpha chain gene; and

(e) human immunodeficiency virus (HIV) nef gene.

6. A recombinant HSV genome according to claim 1 wherein the heterologous gene is inserted in the coding sequence or in a flanking control region of an HSV-1 gene selected from the group of HSV-1 genes consisting of UL23; R11; RL2; UL2; UL3; UL4; UL10; UL11; UL13; UL16; UL20; UL24; UL40; UL41; UL43; UL44; UL45; UL46; UL47; UL50; UL55; UL56; US1; US2; US3; US4; US5; US7; US8; US9; US10; US11; US12; and the locus encoding latency associated transcripts.

7. An HSV mutant containing the genome of claim 1.

8. A method of treating disease comprising administering the recombination HSV genome of any one of claims 1-6 to a subject in need of such therapy.

9. A method of vaccination comprising administering the recombinant HSV genome of any one of claims 1-6 to a subject in need of vaccination.

10. A pharmaceutical composition comprising the recombinant HSV genome of any one of claims 1-6 in admixture with a pharmaceutically acceptable carrier.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 6,613,892 B2
DATED : September 2, 2003
INVENTOR(S) : Preston et al.

Page 1 of 1

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page,

Item [73], Assignee, "**BTG International Inc.**" should read -- **BTG International Limited** --.

Column 17,

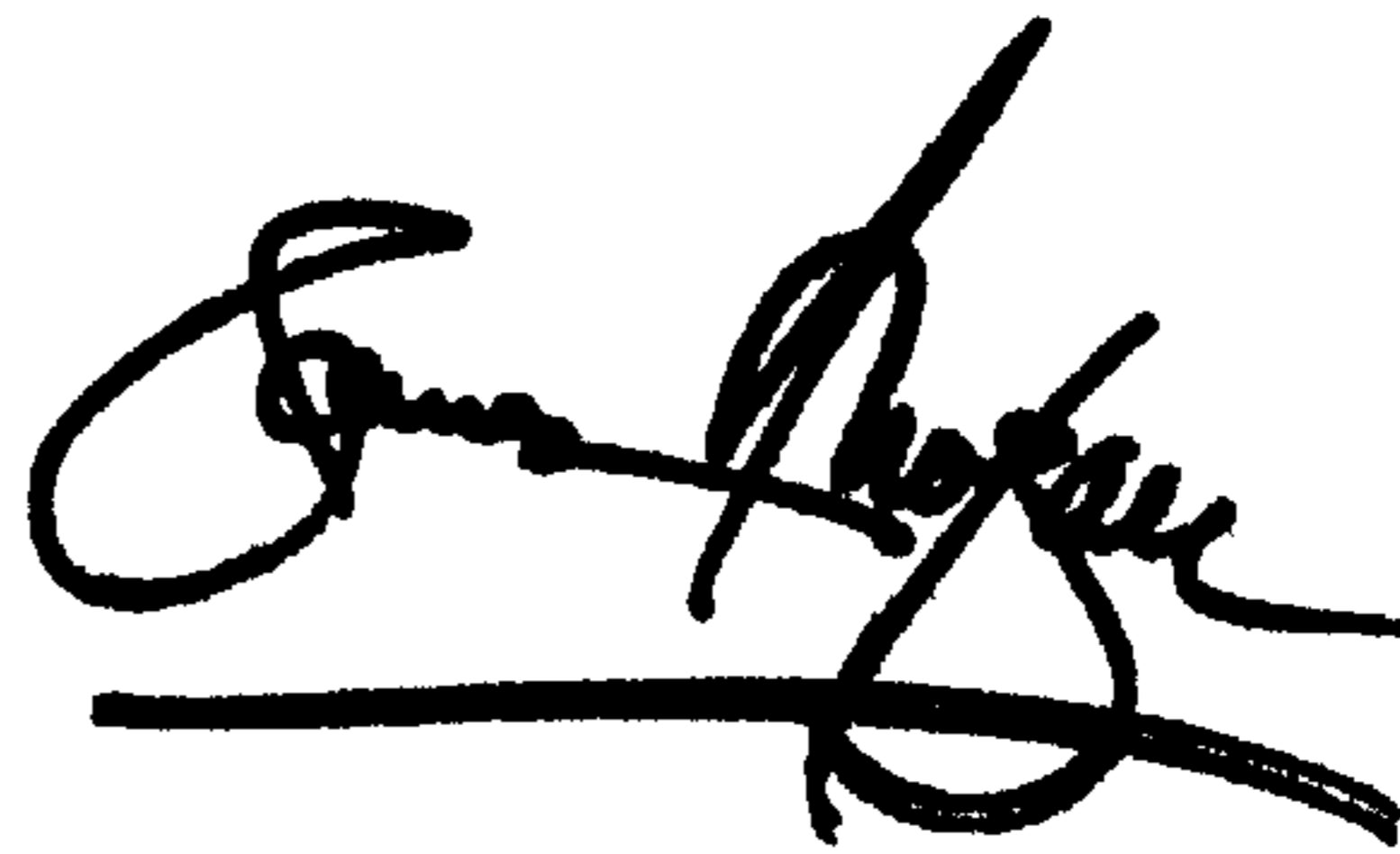
Line 20, "tyro sine" should read -- tyrosine --.

Column 18,

Line 7, "R11" should read -- RL1 --.

Signed and Sealed this

Sixth Day of January, 2004

A handwritten signature in black ink, appearing to read "James E. Rogan", with a horizontal line drawn underneath it.

JAMES E. ROGAN

Director of the United States Patent and Trademark Office